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**(54) A METHOD FOR DETECTING AND/OR QUANTIFYING AND/OR SEPARATING APOPTOTIC
CELLS IN OR FROM A SAMPLE**

**VERFAHREN ZUM NACHWEIS UND/ODER ZUR BESTIMMUNG UND/ODER ZUR ISOLIERUNG
VON APOPTOTISCHEN ZELLEN IN ODER AUS EINER PROBE**

**PROCEDE DE DETECTION ET/OU DE QUANTIFICATION ET/OU DE SEPARATION DE CELLULES
APOPTOTIQUES DANS OU D'UN ECHANTILLON**

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FIELD OF THE INVENTION

The subject invention lies in the field of medicine and pharmacology. In particular, the present invention relates to a procedure to measure apoptosis in physiology and pathology in order to support clinical diagnosis and to evaluate and monitor efficacies of drugs and therapies.

BACKGROUND OF THE INVENTION

The total number of cells of an organism is the result of processes, that lead to the formation of new cells (proliferation) on the one hand and processes, that lead to the breakdown of existing cells (cell death) on the other hand. Apoptosis is the major process responsible for the breakdown of existing cells (1).

Apoptosis is also known as programmed cell death or cell suicide, a process that is characterized by a sequence of distinct events ultimately leading to cell death. Cells, that enter into apoptosis, break up junctions with neighbouring cells if present. The cytoplasm condenses, the nucleus coalesces and breaks up into fragments. At the onset of apoptosis chromatin compacts and segregates against the nuclear envelope. Cytoplasm condenses and the nuclear and cellular membranes begin to convolute. In late stage apoptosis nuclear fragmentation occurs and the cell surface develops protuberances. Apoptotic bodies form that are phagocytosed by adjacent cells. During apoptosis the plasma membrane characteristics alter. For example a modification in carbohydrate composition and a change in membrane hydrophobicity and charge take place. The endoplasmic reticulum transforms into vesicles, that fuse with the plasma membrane and loss of intracellular fluids and ions occurs. In that final stage the cell breaks up into a number of small apoptotic bodies.

Cell death through apoptosis affects single cells within a population in an unsynchronised manner and occurs rather inconspicuously without any inflammatory response (2).

Normal tissue homeostasis requires that for every cell that is added one must die. Apoptosis is an essential process of physiology and proceeds in a well regulated manner in homeostatic balance with its counterpart proliferation (3,4). If the processes of proliferation and apoptosis are out of balance this may result in pathogenesis. It is now becoming evident that an increasing number of pathological situations can be related to aberrant apoptosis. There is currently a lot of research directed at anti-cancer therapies as it is postulated that cancer occurs when cells refuse to die, i.e. when a defect in the apoptotic pathway occurs. An anti-cancer treatment would therefore consist of inducing naturally occurring suicide pathways to yield biotech cancer cures. Apoptosis is furthermore considered to be poten-

tially relevant for a large number of diseases such as ischemia, stroke, heart disease and autoimmunity. It is considered to be such a fundamental biological phenomenon that the challenge is in fact to find a process it is not involved with (13). For example the macrophages dying in the lungs of patients suffering from cystic fibrosis are undergoing apoptosis. The lung-clogging viscosity of the DNA is characteristic of apoptotic death (12). Apoptosis has also been used as diagnostic in autoimmune diseases such as systemic lupus erythematosus (SLE) (13). B-cell malignancies such as B-cell leukemia lymphoma display growth of tumor cells not because of an increased proliferation rate but due to defective apoptosis. Tumor cell metastasis is successful if apoptosis of the migrating tumor cells is suppressed. The ratio between the rate of proliferation and the rate of apoptosis of tumor cells determines the rate of tumor growth and hence its life threatening character.

From these considerations it is clear that modulation of apoptosis in vivo by drugs is a promising strategy for future anti-cancer therapies. In order to diagnose the proliferation: apoptosis ratio in tissue and in particular in tumors and to develop and guide therapies one has to have means to measure apoptosis in a quick and sensitive manner.

To date methods described in the literature fail to serve this purpose as they are elaborate and time consuming or have insufficient specificity.

Duvall et al. (7) describe how apoptotic or non-apoptotic populations can be separated by isopycnic centrifugation using a Percoll density gradient. As these gradients are associated with considerable cell loss comprising up to 60% of the total cells, and they do not remove all Trypan Blue positive cells from the apoptotic population this is a method that is not routinely used (Trypan Blue exclusion is used to determine how many cells are viable).

Fadok et al. (14) describe that the percentage of apoptotic cells in a lymphocyte population was determined by morphologic examination of cyto centrifuged cells stained with Diff-Quick (Baxter Healthcare Corp. Mc. Gaw Park, IL). The key characteristics of apoptosis being nuclear pyknosis and cytoplasmic condensation. Viability of the cells was determined by Trypan Blue exclusion. Fadok et al. (14) describe tests carried out that were directed at discovering how macrophages remove apoptotic lymphocytes by phagocytosis. They illustrated that phagocytosis of apoptotic lymphocytes was inhibited by the L-isomers of compounds structurally related to phosphatidyl serine and that the membranes of apoptotic lymphocytes bound increased amounts of merocyanine 540 dye relative to those of normal cells indicating that their membrane lipids were more loosely packed consistent with a loss of membrane phospholipid asymmetry. The observations suggested that apoptotic lymphocytes lose membrane phospholipid asymmetry and expose phosphatidyl serine on the outer leaflet of the plasma membrane. Macrophages then phago-

cytose an apoptotic lymphocyte after recognition of the exposed phosphatidyl serine. The authors of the document were not concerned with specific detection or isolation of apoptotic cells. They used an aspecific dye for determination of the loose packaging of the membrane apoptotic cells. They discerned the difference between apoptotic and non-apoptotic cells by using isopycnic centrifugation or morphological analysis and followed this by colouring the cells with the aspecific merocyanine 540 dye that binds in a greater amount to loosely packed membranes. No suggestion is given regarding a novel method for specifically discriminating between apoptotic and non-apoptotic cells.

The golden standard up to now for determining the presence of apoptotic cells is the analysis of cellular DNA. For this method the cells are destroyed and the DNA is extracted and analyzed. Tell-tale DNA ladders of about 180 base pairs show up on gels of apoptotic cells. The presence of such ladders in a DNA assay have become the signal for diagnosis of, for example autoimmune diseases such as systemic lupus erythematosus. Analysing DNA fragmentation can be carried out using the diphenylamine reaction of Burton (15) as modified by Sellens and Cohen (16) (6). The long-standing view that double-stranded DNA cleavage was the mechanism that delivered the coupe de grace to apoptotic cells as evidenced by the DNA ladders has been challenged recently. Researchers suggest that cells can undergo apoptosis without internucleosomal cleavage (17) and the implication is that apoptosis could be much more wide-spread than many of the present diagnostic tools suggest.

One of the other big problems in the field of apoptosis is that it is easy to make the mistake of thinking you have inhibited apoptosis if you have killed the cells first as you will not detect apoptotic cells but lysed cells. In looking for compounds or treatments which are capable of inhibiting apoptosis it is essential that the procedure should in fact inhibit apoptosis and not just simply kill the cells by another means before they reach the apoptotic state.

DESCRIPTION OF THE INVENTION

One of the features of apoptotic cells is an altered plasma membrane characteristic (7,8). It has surprisingly been discovered that intact cells, that have entered the program of apoptosis, change their membrane characteristics such that the membranes can bind increased amounts of a reagent having high affinity for phosphatidyl serine. High affinity in this context means having a dissociation constant for phosphatidyl serine $K_d < 10^{-6}$ M, preferably a $K_d < 10^{-10}$ M. Surprisingly good results can be obtained using polypeptides or proteins belonging to the category of the annexins. The light chain of Factor Va is another suitable example of a reagent having high affinity for phosphatidyl serine.

Annexins constitute a well described family of am-

phiphilic proteins, which can bind reversibly to cellular membranes in the presence of cations (9). The primary structure of the annexins comprises a fourfold or eightfold repeated domain, that contains a consensus sequence. A non-conserved and as such an annexin specific N-terminal tail precedes the first domain. The domains are interconnected via short variable linker peptides. Annexin proteins can be purified from tissues like placental tissue (10) or can be obtained via recombinant techniques (11).

In WO 91/09628 annexins are described for binding to phosphatidyl serine on blood platelets that have entered the prothrombin activation state. Nothing is taught or suggested about using annexin for any other objective and no suggestion is given regarding the use in a method according to the invention.

The observation, that apoptotic cells have an increased number of binding sites for annexins has lead to the development of assays, that discriminate rapidly between non-apoptotic and apoptotic cells on an individual basis without destroying the cells. The procedure is based on the following principle. Suspended cells or tissue sections can be mixed with a reagent specific for phosphatidyl serine and the amount of cell surface bound reagent can be measured either directly by virtue of a label conjugated to the reagent or indirectly via antibodies specific for the reagent. The parameter of cell-bound reagent discloses whether the cell is apoptotic or not. The determination can be of a qualitative or a quantitative nature.

It is also possible to separate apoptotic cells from non-apoptotic cells through selection of a marker conjugated to the reagent, which is suited for such a separation process. A number of markers suitable for selecting a specific population and isolating it from a different population are known in the state of the art.

In general the subject invention is directed at a method for detecting and/or optionally quantifying apoptotic cells in a sample comprising

a) contacting the sample with a detectable reagent having high affinity for phosphatidyl serine, high affinity throughout the description means having a dissociation constant for phosphatidyl serine with $K_d < 10^{-6}$ M, and

b) qualitatively and/or optionally quantitatively detecting intact cells that have reacted with the detectable reagent having high affinity for phosphatidyl serine. This method can also be used as part of a method for separating apoptotic cells in a sample. The method directed at separation of apoptotic cells is carried out by

a) contacting the sample with a detectable reagent having high affinity for phosphatidyl serine and
b) qualitatively and/or optionally quantitatively detecting intact cells that have reacted with the detectable reagent having high affinity for phosphatidyl serine, said detection occurring before or after step

c) for separating apoptotic cells from non-apoptotic cells, which separation can occur due to the fact that apoptotic cells have been provided in step a) with the detectable reagent having high affinity for phosphatidyl serine, said detectable reagent also being selectable in a manner known per se. The detection of intact cells as opposed to cells that have undergone lysis can be carried out in a manner known per se. It is known for example that propidiumiodide is a label that recognizes cells that have undergone lysis but does not recognize intact cells. After treatment with propidiumiodide the intact cells can also be separated from cells that have undergone lysis in a manner known per se, such as through flow cytometry. In general a label that does not bind to intact cells but does bind to cells that have undergone lysis can be used in a manner known per se to discern lysed from non lysed cells. Preferably the label for lysed cells is also discernible from the marker for the reagent having high affinity for phosphatidyl serine and can also be used to actually separate a population of lysed cells from cells that are intact.

As stated above the reagent having high affinity for phosphatidyl serine can be a polypeptide or protein classified as an annexin. The specific phospholipid-binding properties of annexin in general and of annexin V in particular in combination with the ability to conjugate an annexin with a wide range of compounds in such a manner that the conjugate still possesses phospholipid-binding properties can be used to determine qualitatively and/or quantitatively cell apoptosis either occurring spontaneously or induced by any kind of environmental factor. Any derivative of an annexin still exhibiting a high affinity for phosphatidyl serine can also be used in a method according to the invention. When using an annexin or a derivative thereof as the reagent having high affinity for phosphatidyl serine in a method according to the subject invention it is preferable to also contact the sample with the reagent having high affinity for phosphatidyl serine in the presence of a cation, preferably in the presence of a bivalent cation in order for the reagent to exhibit binding capacity with phosphatidyl serine. The bivalent cation can be selected for example from the group comprising Cd^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} and preferably Ca^{2+} . In the presence of certain amounts of Ca^{2+} , for example annexin V adsorbs to phospholipid membranes in an extrinsic fashion. The Ca^{2+} ions are believed to bridge between the protein molecule and the phospholipid membrane by binding to sites in annexin V and ligating to 6 carbonyl oxygens of specific amino acids. The thus chelated Ca^{2+} ions however are unsaturated by coordination and charge. The 7th coordination site may then accept the phosphoryl moiety of phosphatidyl serine. The bridging appears to be very stable under physiological ionic strength and pH conditions and above threshold Ca^{2+} levels but is disjointed rapidly and completely upon decreasing the Ca^{2+} concentration below thresh-

old level. Interestingly these threshold levels of Ca^{2+} appear to be inversely related to the phosphatidyl serine content of the membrane. Annexin V in particular displays a high affinity for phosphatidyl serine containing membranes. Expression of the affinity with an objective parameter such as a K_d appears however to be difficult and is given as an estimation to be less than 10^{-10} M. In practice this is reflected by the fact that no other phospholipid-binding proteins like the vitamin K dependent coagulation factors and the cofactors VII (a) and V(a) seriously compete with annexin V for binding to a membrane.

It is also possible to carry out the method according to the invention using a combination of two or more different cations. In particular the combination of Ca^{2+} and Zn^{2+} gives good results. It is also possible to use a trivalent cation, such as Tb^{3+} .

The reagent to be used must have a high affinity for phosphatidyl serine. It must also preferably not have such a high affinity for other membrane components that are available on the outer leaflet of cell membranes of non-apoptotic cells. In particular it should not bind very strongly to phosphatidylcholine or sphingomyelin and preferably should not bind very strongly to other outer membrane components of non-apoptotic cells. When the method according to the invention is to be used for comparative purposes merely to determine a qualitative effect this latter requirement is not stringent as one merely has to differentiate between the cells to which a large amount of reagent has been bound and cells to which a smaller amount of reagent has been bound. Naturally however the higher the affinity of the reagent is for phosphatidyl serine the better. Differentiation between the various phospholipids that are comprised in the outer membranes of non-apoptotic and apoptotic cells is what makes annexins ideal reagents for separating apoptotic cells from non-apoptotic cells. Any reagent that can specifically determine between phosphatidyl serine and phosphatidylcholine in favour of phosphatidyl serine can be used in a method according to the invention. An additional characteristic of the reagent having high affinity for phosphatidyl serine is that it can still be capable of binding phosphatidyl serine when it is conjugated to the label to be used for detection and/or selection.

The subject method now offers the means to analyse the results of certain treatments and/or compounds on apoptotic cells and also on the formation of apoptotic cells. It now becomes possible to evaluate the effects of the treatment and/or of compounds on the activation or inactivation of the pathway leading to apoptotic cells. It also makes a simple separation of apoptotic cells from non-apoptotic cells in a certain population possible without having to destroy the cells. The subsequently separated populations of apoptotic and non-apoptotic cells can then therefore also be used for further tests and/or clinical applications like transplantation of autologous or heterologous stem cells or bone marrow cells. It is also

possible to analyse and isolate on an individual cell basis. The method according to the invention can be used on a sample of suspended cells or tissue sections.

The amount of reagent having high affinity for phosphatidyl serine bound per cell can be measured either directly or indirectly. In the case of a direct measurement the cells to be analysed are contacted with reagent having high affinity for phosphatidyl serine that carries a label. Such a label can be any generally acceptable detectable marker such as a fluorescent marker, a radioactive marker, an enzyme, a metal, a dye, a detectable immunoglobulin or a protein part. Suitable examples are radioactive marker selected from the group comprising ^{125}I , ^{131}I , ^{111}In , ^{32}P , ^{35}S and ^{99}Tc . Suitable fluorescent markers can be selected from the group comprising fluorescein, phycoerythrin and rhodamin. Suitable enzyme markers can be selected from the group comprising alcohol dehydrogenase, peroxidase and alkaline phosphatase. The protein part can be selected from the group ferritin, biotin, avidin and streptavidin and derivatives thereof. A suitable dye can be selected from the group comprising Evans Blue and Coomassie Brilliant Blue. Labeling procedures are specific for the type of label and are described in the literature or are provided by the commercial suppliers of the labels. The above-mentioned examples of labels are merely illustrations and any generally acceptable label that is commercially available can be used.

For an indirect measurement the sample to be tested can be contacted with unlabeled reagent having high affinity for phosphatidyl serine and the amount of bound reagent can be determined by reagent specific antibodies in a manner known per se.

Measurement of apoptosis can be performed after contacting the sample with cations and an annexin protein for example. The measurement of apoptosis can be carried out using standard techniques for the determination of the presence and/or amount of label. Examples of techniques that can be used for the analyses are flow- and image cytometry and image analysis. In the case of indirect measurement using antibodies any antibody detection immunoassays can be used that are described in the state of the art.

In particular the separation of apoptotic cells from a sample can occur using a reagent having high affinity for phosphatidyl serine conjugated to a fluorescent label followed by use of a FACS cell sorter system set to separate cells with a large amount of fluorescence from cells with low fluorescence or even no fluorescence. The separation can also occur through use of a solid phase that exhibits a high affinity for the reagent having high affinity for phosphatidyl serine or the conjugate form of the reagent that is used. For example immobilised biotin can be used to bind streptavidin conjugated to the reagent having high affinity for phosphatidyl serine.

The method according to the invention can be used for determining the effect of a compound or a specific treatment on the degree of apoptosis in an individual or

particular sample. This determination of the effect can be ascertained by carrying out the method according to the invention with a sample that has been subjected to the presence of the compound to be tested or has been subjected to the treatment which is to be tested and comparing this result to the result obtained carrying out the method according to the invention under the same conditions with a standard sample or with a sample taken prior to the presence of the compound and/or prior to subjecting the sample to the treatment to be tested. This comparison can be of a qualitative or a quantitative nature.

A kit suitable for carrying out the method according to the invention also falls within the scope of the invention. Such a kit must comprise a reagent specific for phosphatidyl serine that is detectable or that can be made detectable even when the reagent is conjugated to phosphatidyl serine. High affinity in this context means having a dissociation constant for phosphatidyl serine with $K_d < 10^{-6}\text{M}$. The reagent can already have been provided with a marker in the kit or a marker can also be provided with the kit. The kit must also comprise a detectable and preferably selectable label capable of discerning between intact cells and cells that have undergone lysis. A suitable example of such a label is propidiumiodide. Optionally the cation required for binding the reagent specific for phosphatidyl serine to the cell membrane can be included when the kit comprises an annexin as specific reagent for phosphatidyl serine. The kit can furthermore comprise media suitable for carrying out the method according to the invention. The kit can also comprise standard samples of specific cell populations which can be used in a comparative assay. The kit can be specific for the type of cell or the type of tissue that is to be analysed due to the presence of suitable media and/or labels for these specific cells. In the case of analyses of effects of pharmaceuticals for cancer treatment it is quite often the case that specific cancers are specific for certain tissues or cell types and in such cases it is interesting to use specific media or markers suitable for such cells.

EXAMPLE 1

Neutrophils are isolated from blood by standard techniques. The neutrophils are kept overnight in culture medium in the absence or presence of cytokines. Cytokines are known to prevent neutrophils from entering apoptosis.

In order to measure apoptosis in the neutrophil population, the cells are mixed with 3 mM Ca^{2+} in buffered saline and 10 $\mu\text{g/ml}$ final concentration of annexin V conjugated with fluorescein and 10 $\mu\text{g/ml}$ propidiumiodide. The mixed cells are analysed by flow cytometry. Figure 1 illustrating results of the test without addition of cytokines and Figure 2 illustrating results of the test with cytokines illustrate that the propidiumiodide negative cells contain 2 populations that are distinguishable

by this method. A population of intact cells with a low amount of bound annexin V and a population of intact cells with a high amount of bound annexin V. The latter population appeared apoptotic as revealed by other analytical methods. From the flow cytometric analysis it followed that in the absence of cytokines the ratio of non-apoptotic cells to apoptotic cells is 1.82:1, whereas in the presence of cytokines this ratio is 7.62:1. This illustrates the fact that due to the cytokine presence the amount of cells entering the apoptotic phase is markedly lower. The effect of a different compound than a cytokine can be compared in a similar manner and thus the effect of such a compound on apoptosis can also be discerned rapidly and sensitively.

EXAMPLE 2

The wells of a microtitre plate are seeded with adherent cells in a manner known per se. The selection of the adherent cell type to be used will depend on the diagnosis to be carried out. A number of different cell types that are suitable are commercially available and the selections of the type to be used will be apparent to a person skilled in the art. The cells are subsequently treated in a manner potentially resulting in induction or inhibition of apoptosis. The extent of apoptosis is then assessed by addition of Annexin V e.g. conjugated with a reporter group such as fluorescein or biotin in a buffer containing Ca^{+2} ions. After incubation the unbound Annexin V is washed away. The amount of bound Annexin V is then directly or indirectly measured. Direct measurement can occur in the case of Annexin V-fluorescein in a microplate reader. Indirect measurement can occur using streptavidinhorseradishperoxidase and a chromogenic substrate in combination with Annexin V-biotin. The amount of bound Annexin indicates the extent of apoptosis per well.

EXAMPLE 3

The present invention will hereinafter be described by examples as a tool to serve important clinical questions in the therapy of various diseases. Example 3.IV is a comparative example which falls outside the scope of the claims.

3.1. Evaluation of the efficacy of an anti-cancer therapy for a leukemic patient.

Blood is withdrawn from the patient under conditions that are not fatal to the white blood cells. Such conditions are described by public literature.

The blood is centrifuged to form a buffy coat, which contains the white blood cells. These are collected and centrifuged by density centrifugation e.g. Ficoll or Percoll of a desired density. The density chosen depends on the type of leukemia. The procedure is constructed to isolate highly enriched leukemic cells. The isolated

leukemic cells are suspended in culture medium e.g. Iscove's DMEM, or RPMI1640. The medium can be supplemented with serum of human or bovine origin and with additives like glutamic acid, growth factors, cytokines or other modulators of cell physiology.

The suspended cells are placed in culture dishes and incubated at e.g. 37°C. The main components of possible anti-cancer therapies, like e.g. fludarabine, methotrexate, cyclosporine, are added in varying concentrations to the cells in culture. The cells are cultured further in the presence of the components. At predetermined time points samples are withdrawn and measured for cell death by the principle of the method of the present invention: A cell sample is washed with Hepes/NaCl buffer containing 0.5-5 mM Ca^{+2} -ions and finally suspended in 445 μl buffer containing approximately 10^4 - 10^6 cells. 5 μl of Annexin V, conjugated with fluorescein, and 50 μl of propidium iodide are added to the sample resulting in e.g. 0.025 - 10 $\mu\text{g/ml}$ and 0.5 - 20 $\mu\text{g/ml}$ respectively. The sample is further incubated and then analysed by flow cytometric two-color analysis according to established procedures.

Figure 3 illustrates a typical outcome of such analysis. This analysis gives the population distribution of viable, apoptotic and necrotic cells. The fraction of dead cells (apoptotic + necrotic cells) is indicative for the efficacy of the drug used on this particular cell type. Hence, this assay reveals drug resistency of the leukemic cells of the patient and, thus guides the clinician in selecting an anti-cancer therapy.

3. II. Evaluation of the progression of AIDS.

Blood is withdrawn from a HIV-positive subject or an AIDS patient under conditions that are not fatal to the white blood cells. Such conditions are described by public literature. The peripheral blood mononuclear cells (PBMC) are isolated by standard techniques including centrifugation to obtain a buffy coat and subsequently centrifugation of the buffy coat on a density gradient like e.g. Ficoll® or Percoll® of a desired density. The PBMC are suspended in culture medium e.g. Iscove's DMEM, or RPMI1640. The medium can be supplemented with serum of human or bovine origin and with additives like glutamic acid, growth factors, cytokines or other modulators of cell physiology. The suspended cells are placed in culture dishes and incubated at e.g. 37°C. At predetermined time points samples are withdrawn and measured for cell death by the principle of the method of the present invention. The cells are washed with phosphate buffered saline and then incubated with phycoerythrin labelled CD4 or CD8 antibody. The cells are then washed with Hepes/NaCl, containing 0.5-5 mM Ca^{+2} -ions. The cells are suspended in 445 μl of buffer containing e.g. 10^4 - 10^6 cells. 5 μl of Annexin V, conjugated with fluorescein, and 50 μl of propidium iodide are added to the sample resulting in 0.025 - 10 $\mu\text{g/ml}$ and 0.5 - 20 $\mu\text{g/ml}$ respectively. The sample is further incubated and

then analysed by flow cytometric three-color analysis according to established procedures. Figure 3 illustrates a typical outcome of such analysis. This analysis gives the population distribution of viable, apoptotic and necrotic cells. Because of the three-color analysis it is possible to measure the percentage of death cells in the PMBC subsets CD4+ and CD8+. The percentage of death cells in these subpopulations of PBMC is indicative for the progression of AIDS and guides the clinician in the selection of therapy.

3. III. Evaluation of the status of the disease systemic lupus erythematosus.

Blood is withdrawn from SLE patients and treated to obtain PMBC as described in example 3.II. The isolated PMBC are suspended in culture medium like p.e. Iscove's DMEM, or RPMI 1640. The medium can be supplemented with serum of human or bovine origin and with additives like glutamic acids, growth factors, cytokines or other modulators of cell physiology. The suspended cells are placed in culture dishes and incubated at e.g. 37°C. At predetermined time points samples are withdrawn and measured for cell death by the principle of the method of the present invention.

The cells are then washed with HEPES/NaCl, containing 0.5-5 mM Ca²⁺-ions. The cells are suspended in 445 µl of buffer containing e.g. 10⁴-10⁶ cells. 5 µl of Annexin V, conjugated with fluorescein, and 50 µl of propidium iodide are added to the sample resulting in e.g. 0.025 - 10 µg/ml and 0.5 - 20 µg/ml respectively. The sample is further incubated and then analysed by flow cytometric two-color analysis according to established procedures.

Figure 3 illustrates a typical outcome of such analysis. This analysis gives the population distribution of viable, apoptotic and necrotic cells. The fraction of death cells (apoptotic + necrotic cells) is indicative for the status of the activity of the disease and, hence, guides the clinician in selecting a method of treatment.

3.IV. Evaluation of normal or aberrant development of the embryo.

Pregnant mice, from 11-13 days post conception (plug = day 0), are sacrificed by cervical dislocation after anesthesia with ether. The uterus is taken out, embryos are collected and divided into two groups. Embryos are temporarily cultured for detection of cell death by injection of Annexin V, conjugated with biotin. Injections are into the ventricle of the heart using a Hamilton-Syringe pipetting system with glass needles with a tip diameter of 15-25 µm. A volume of approximately 3 µm Annexin V-biotin solution is injected under a surgical microscope while the embryo is kept in buffer of 37°C. When successfully injected, a temporary blanching of the umbilical vein will be seen. Heart activity is examined and embryos that survive a 30 minute period of incubation are fur-

ther processed. After incubation embryos are fixed overnight in 4% Forman heaps buffer for Light Microscopy (LM) or in 2% glutaraldehyde-2% paraformaldehyde cacodylate buffer for Electron Microscopy at 4°C.

Histological Analysis for LM

Following fixation embryos are dehydrated, embedded in paraffin and serially sectioned at 3 µm. Endogenous peroxidase is blocked by incubation in methanol/H₂O₂ (9:1 v/v) for 20 minutes. Sections are washed in phosphate buffered saline (PBS). Cell bound Annexin V-biotin is detected using the avidin-biotin complex method with horseradish peroxidase conjugated avidin (avidin-HRP) (Vector ABC Elite kit, Brunschwig, Germany) at room temperature. Staining is developed with 3,3'-diaminobenzidinetetrahydrochloride (DAB) and counterstained with haematoxylin.

Histological Analysis for EM.

After incubation with Annexin V-biotin, embryos are fixed by intra cardiac injection, using glass needles as previously described. Then tissue segments are removed and fixed overnight at 4°C. Sections of 100 µm are cut on a vibratome and staining of Annexin V-biotin is developed as for LM. After DAB, sections are post-fixed with OsO₄ and stained with 3% uranylacetate in toto followed by dehydration and embedding in plastic (Durcupan). Tissue is cut in ultrathin slices on a microtome (Reichert Jung Ultracut S) and finally stained with 1% lead citrate. Sections are examined by electron microscopy.

Annexin V-biotin binds to dying or death cells and not to viable cells. Hence, the sections of the treated embryo will visualise the topology of cell death in the developing embryo at the time point of removal of the embryo's from the uterus. Cell death is programmed in the embryo by coordinates of space and time. Cell death aberrant by space and time will have consequences for the organism. Spina bifida e.g. is the result of the lack of cell death at a certain location at a certain time point. The inventory of normally programmed cell death is well described by public literature. This inventory functions as reference to judge the (ab)normality of the cell death in the developing embryo as measured by the method described in this example. Measurement of cell death in the developing embryo is of importance in studying genetic defects and the evaluation of the teratogenicity of compounds.

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Claims

1. A method for detecting and/or quantifying and/or isolating apoptotic cells in a sample, comprising

a) contacting the sample with a detectable high-affinity reagent having a dissociation constant for phosphatidyl serine with $K_d < 10^{-6}$ M and

b) qualitatively and/or quantitatively detecting cells that have reacted with the detectable reagent having high affinity for phosphatidyl serine, said detection occurring before or after the optional isolation step c)

c) isolating apoptotic cells from non-apoptotic cell on the basis of the apoptotic cells being bound to the said detectable reagent in step a) said detectable reagent also being selectable.

2. A method according to claim 1 wherein the cells are intact cells.

3. A method according to claim 1, wherein the reagent having high affinity for phosphatidyl serine is a polypeptide or protein classified as an annexin.

4. A method according to any of the preceding claims, wherein the cells can be distinguished into cells that have undergone lysis and intact cells through use of a label for detecting cells that have undergone lysis.

5. A method according to claim 4 wherein the label is propidiumiodide.

6. A method according to claim 4 or 5 wherein the cells are detected through flow cytometry.

7. A method according to any of the preceding claims, wherein the said detectable reagent having high affinity for phosphatidyl serine is annexin V or a derivative thereof.

8. A method according to any of the preceding claims, wherein the reagent having high affinity for phosphatidyl serine is contacted with the sample in the presence of a cation in order for the reagent to exhibit affinity for phosphatidyl serine.

9. A method according to claim 8 wherein the cation is bivalent.

10. A method according to claim 9, wherein the bivalent cation is selected from the group comprising Cd²⁺, Zn²⁺, Mn²⁺, Co²⁺, and Ca²⁺.

11. A method according to claim 10 wherein the cation is Ca²⁺.

12. A method according to any of claims 8-11, wherein the reagent having high affinity for phosphatidyl serine is contacted with the sample in the presence of both Ca²⁺ and Zn²⁺ in order for the reagent to exhibit high affinity for phosphatidyl serine.

13. A method according to claim 8, wherein the cation is Tb³⁺.

14. A method according to any of the preceeding claims, wherein the reagent specific for phosphatidyl serine is provided with a detectable marker.
15. A method according to claim 14 wherein the detectable marker is selected from the following: A fluorescent marker, a radioactive marker, an enzyme, a metal, a dye, a detectable immunoglobulin, or a protein part.
16. A method according to claim 14 or 15, wherein the radioactive marker is selected from the group comprising ^{125}I , ^{131}I , ^{111}In , ^{32}P , ^{35}S , and ^{99}Tc .
17. A method according to claim 14 or 15, wherein the fluorescent marker is selected from the group comprising fluorescein, phycoerythrin and rhodamin.
18. A method according to claim 14 or 15, wherein the enzyme is selected from the group comprising alcohol dehydrogenase, peroxidase and alkaline phosphatase.
19. A method according to claim 14, wherein the detectable marker is selected from the group comprising ferritin, biotin, avidin and streptavidin and derivatives thereof.
20. A method according to claim 14 or 15, wherein the dye is selected from the group comprising Evans Blue and Coomassie Brilliant Bleu.
21. A method according to any of claims 1-20, wherein the apoptotic cells are isolated through use of a flow cytometer or antibody detection immunoassay.
22. A method according to any of claims 1-20, wherein apoptosis is measured in a microtitre plate provided with adherent cells.
23. A method according to any of claims 1-20, wherein the apoptotic cells are separated from non-apoptotic cells through use of a solid phase that exhibits a high affinity for the reagent that is bound to the apoptotic cell.
24. A method according to any of claims 1-20 wherein the apoptotic cells are separated from non-apoptotic cells through use of a solid phase that exhibits a high affinity for annexin or a conjugated form of annexin.
25. A method for determining the effect of a compound or a specific treatment on the degree of apoptosis in an individual and/or a sample comprising carrying out the method according to any of the preceeding claims with a sample that has been subjected to the presence of the compound and/or the specific treatment to be tested and comparing the result to the result obtained carrying out the method according to the preceeding claims under the same conditions with a standard sample and/or with a sample taken prior to the presence of the compound and/or the specific treatment to be tested.
26. A kit suitable for carrying out the method according to any of the preceeding claims, comprising a reagent having a dissociation constant for phosphatidyl serine with $K_d < 10^{-6}$ M and is detectable or can be made detectable, said kit further comprising a label for distinguishing cells that have undergone lysis from intact cells.
27. A kit according to claim 26 wherein the label is propidiumiodide.
28. A kit according to claim 26 or 27, further comprising a cation.
29. A kit according to any of claims 26-28 further comprising suitable media.
30. A kit according to any of claims 26-29 wherein the label is selectable such that lysed and intact cells can be separated.
31. Use of a method according to any of the claims 1-22 and/or a kit according to any of claims 26-30 for separating suitable cells for transplant from unsuitable cells.
32. Use of a method according to claim 31 for separating cells for transplantation the cells being autologous or heterologous stem cells or bone marrow cells.

40 Patentansprüche

1. Verfahren zum Nachweis und/oder zur Quantifizierung und/oder zur Isolierung apoptotischer Zellen in einer Probe, umfassend
 - a) Inkontaktbringen der Probe mit einem nachweisbaren hochaffinen Reagenz mit einer Dissoziationskonstante für Phosphatidylserin K_d von $< 10^{-6}$ M und
 - b) qualitativ und/oder quantitativ Nachweisen von Zellen, die mit dem nachweisbaren Reagenz, das eine hohe Affinität für Phosphatidylserin aufweist, reagiert haben, wobei der Nachweis durchgeführt wird vor oder nach dem fakultativen Isolierschritt c)
 - c) Isolieren apoptotischer Zellen von nicht-apo-

- ptotischen Zellen auf Basis der in Schritt a) an das nachweisbare Reagenz gebundenen apoptotischen Zellen, wobei das nachweisbare Reagenz auch selektierbar ist.
2. Verfahren nach Anspruch 1, wobei die Zellen intakte Zellen sind. 5
 3. Verfahren nach Anspruch 1, wobei das Reagenz mit hoher Affinität für Phosphatidylserin ein als Annexin zu klassifizierendes Polypeptid oder Protein ist. 10
 4. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Zellen unterschieden werden können in Zellen, die Lyse eingegangen sind, und intakte Zellen, durch Verwendung einer Markierung zum Nachweis von Zellen, die Lyse eingegangen sind. 15
 5. Verfahren nach Anspruch 4, wobei die Markierung Propidiumjodid ist. 20
 6. Verfahren nach Anspruch 4 oder 5, wobei die Zellen durch Durchflußcytometrie nachgewiesen werden. 25
 7. Verfahren nach einem der vorhergehenden Ansprüche, wobei das nachweisbare Reagenz mit hoher Affinität für Phosphatidylserin Annexin V oder ein Derivat davon ist. 30
 8. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Reagenz mit hoher Affinität für Phosphatidylserin mit der Probe in der Gegenwart eines Kations in Kontakt gebracht wird, damit das Reagenz Affinität für Phosphatidylserin aufweist. 35
 9. Verfahren nach Anspruch 8, wobei das Kation ein zweiwertiges Kation ist. 40
 10. Verfahren nach Anspruch 9, wobei das zweiwertige Kation ausgewählt wird aus der Gruppe umfassend Cd^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} und Ca^{2+} . 45
 11. Verfahren nach Anspruch 10, wobei das Kation Ca^{2+} ist. 50
 12. Verfahren nach einem der Ansprüche 8-11, wobei das Reagenz mit hoher Affinität für Phosphatidylserin mit der Probe in der Gegenwart von sowohl Ca^{2+} als auch Zn^{2+} in Kontakt gebracht wird, damit das Reagenz hohe Affinität für Phosphatidylserin aufweist. 55
 13. Verfahren nach Anspruch 8, wobei das Kation Tb^{3+} ist.
 14. Verfahren nach einem der vorhergehenden Ansprüche, wobei das für Phosphatidylserin spezifische Reagenz mit einem nachweisbaren Marker versehen ist.
 15. Verfahren nach Anspruch 14, wobei der nachweisbare Marker ausgewählt wird aus den nachfolgenden: einem Fluoreszenzmarker, einem radioaktiven Marker, einem Enzym, einem Metall, einem Farbstoff, einem nachweisbaren Immunglobulin oder einem Proteinanteil.
 16. Verfahren nach einem der Ansprüche 14 oder 15, wobei der radioaktive Marker ausgewählt wird aus der Gruppe umfassend ^{125}I , ^{131}I , ^{111}In , ^{32}P , ^{35}S und ^{99}Tc .
 17. Verfahren nach Anspruch 14 oder 15, wobei der Fluoreszenzmarker ausgewählt wird aus der Gruppe umfassend Fluorescein, Phycoerythrin und Rhodamin.
 18. Verfahren nach Anspruch 14 oder 15, wobei das Enzym ausgewählt wird aus der Gruppe umfassend Alkoholdehydrogenase, Peroxidase und alkalische Phosphatase.
 19. Verfahren nach Anspruch 14, wobei der nachweisbare Marker ausgewählt wird aus der Gruppe umfassend Ferritin, Biotin, Avidin und Streptavidin und Derivate davon.
 20. Verfahren nach Anspruch 14 oder 15, wobei der Farbstoff ausgewählt wird aus der Gruppe umfassend Evans Blau und Coomassie Brilliantblau.
 21. Verfahren nach einem der Ansprüche 1-20, wobei die apoptotischen Zellen isoliert werden durch die Verwendung eines Durchflußcytometers oder eines Immunoassays zum Antikörnernachweis.
 22. Verfahren nach einem der Ansprüche 1-20, wobei Apoptose gemessen wird in einer Mikrotiterplatte, die mit adhären Zellen versehen ist.
 23. Verfahren nach einem der Ansprüche 1-20, wobei die apoptotischen Zellen von nicht-apoptotischen Zellen abgetrennt werden durch Verwendung einer Festphase, die eine hohe Affinität für das an die apoptotischen Zellen gebundene Reagenz aufweist.
 24. Verfahren nach einem der Ansprüche 1-20, wobei die apoptotischen Zellen von nicht-apoptotischen Zellen abgetrennt werden durch Verwendung einer Festphase, die eine hohe Affinität für Annexin oder eine Konjugatform von Annexin aufweist.
 25. Verfahren zur Bestimmung der Wirkung einer Verbindung oder einer spezifischen Behandlung auf

das Ausmaß von Apoptose in einem Individuum und/oder in einer Probe, umfassend das Ausführen des Verfahrens nach einem der vorhergehenden Ansprüche mit einer Probe, die der Gegenwart der zu untersuchenden Verbindung und/oder der zu untersuchenden spezifischen Behandlung ausgesetzt worden ist, und das Vergleichen des Ergebnisses mit dem Ergebnis, das erhalten wurde durch Ausführen des Verfahrens nach einem der vorhergehenden Ansprüche unter den gleichen Bedingungen mit einer Standardprobe und/oder mit einer Probe, die vor der Gegenwart der zu untersuchenden Verbindung und/oder der zu untersuchenden spezifischen Behandlung entnommen worden ist.

26. Kit, geeignet zum Ausführen des Verfahrens nach einem der vorhergehenden Ansprüche, umfassend

ein Reagenz, das eine Dissoziationskonstante für Phosphatidylserin K_d von $< 10^{-6}$ M aufweist und nachweisbar ist oder nachweisbar gemacht werden kann,

wobei das Kit weiter eine Markierung umfaßt, zur Unterscheidung von Zellen, die Lyse eingegangen sind, von intakten Zellen.

27. Kit nach Anspruch 26, wobei die Markierung Propidiumjodid ist.

28. Kit nach Anspruch 26 oder 27, weiterhin umfassend ein Kation.

29. Kit nach einem der Ansprüche 26-28, weiterhin umfassend ein geeignetes Medium.

30. Kit nach einem der Ansprüche 26-29, wobei die Markierung selektierbar ist, so daß lysierte und intakte Zellen getrennt werden können.

31. Verwendung eines Verfahrens nach einem der Ansprüche 1-22 und/oder eines Kits nach einem der Ansprüche 26-30 zum; Abtrennen von für ein Transplantat geeigneten Zellen von ungeeigneten Zellen.

32. Verwendung eines Verfahrens nach Anspruch 31 zum Abtrennen von Zellen für eine Transplantation, wobei die Zellen autologe oder heterologe Stammzellen oder Knochenmarkszellen sind.

Revendications

1. Procédé pour détecter et/ou quantifier et/ou isoler des cellules apoptotiques dans un échantillon, qui consiste :

a) à mettre en contact l'échantillon avec un réactif détectable à haute affinité, ayant une constante de dissociation vis-à-vis de la phosphatidylsérine $K_d < 10^{-6}$ M, et

b) à détecter qualitativement et/ou quantitativement les cellules qui ont réagi avec le réactif détectable ayant une grande affinité pour la phosphatidylsérine, ladite détection ayant lieu avant ou après l'étape éventuelle d'isolement c),

c) à isoler les cellules apoptotiques des cellules non apoptotiques sur la base du fait que les cellules non apoptotiques sont fixées audit réactif détectable dans l'étape a), ledit réactif détectable étant aussi sélectionnable.

2. Procédé selon la revendication 1, dans lequel les cellules sont des cellules intactes.

3. Procédé selon la revendication 1, dans lequel le réactif ayant une grande affinité pour la phosphatidylsérine est un polypeptide ou une protéine classée en tant qu'annexine.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel les cellules peuvent être subdivisées en cellules ayant subi une lyse et en cellules intactes, par utilisation d'un marqueur destiné à détecter les cellules ayant subi une lyse.

5. Procédé selon la revendication 4, dans lequel le marqueur est l'iodure de propidium.

6. Procédé selon la revendication 4 ou 5, dans lequel les cellules sont détectées par cytométrie d'écoulement.

7. Procédé selon l'une quelconque des revendications précédentes, dans lequel ledit réactif détectable ayant une grande affinité pour la phosphatidylsérine est l'annexine V ou l'un de ses dérivés.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel le réactif ayant une grande affinité pour la phosphatidylsérine est mis en contact avec l'échantillon en présence d'un cation, pour que le réactif déploie son affinité pour la phosphatidylsérine.

9. Procédé selon la revendication 8, dans lequel le cation est bivalent.

10. Procédé selon la revendication 9, dans lequel le cation bivalent est choisi dans le groupe comprenant Cd^{2+} , Zn^{2+} , Mn^{2+} , CO_2^{+} et Ca^{2+} .

11. Procédé selon la revendication 10, dans lequel le cation est Ca^{2+} .

12. Procédé selon l'une quelconque des revendications 8 à 11, dans lequel le réactif ayant une grande activité pour la phosphatidylsérine est mis en contact avec l'échantillon en présence des deux cations Ca^{2+} et Zn^{2+} pour que le réactif déploie sa grande affinité pour la phosphatidylsérine. 5
13. Procédé selon la revendication 8, dans lequel le cation est Tb^{3+} .
14. Procédé selon l'une quelconque des revendications précédentes, dans lequel le réactif spécifique de la phosphatidylsérine est pourvu d'un marqueur détectable. 10
15. Procédé selon la revendication 14, dans lequel le marqueur détectable est choisi parmi ce qui suit : un marqueur fluorescent, un marqueur radioactif, une enzyme, un métal, un colorant, une immunoglobuline détectable, ou un élément protéique. 15
16. Procédé selon la revendication 14 ou 15, dans lequel le marqueur radioactif est choisi dans le groupe comprenant les isotopes ^{125}I , ^{131}I , ^{111}In , ^{32}P , ^{35}S et ^{99}Tc . 20
17. Procédé selon la revendication 14 ou 15, dans lequel le marqueur fluorescent est choisi dans le groupe comprenant la fluorescéine, la phycoérythrine et la rhodamine. 25
18. Procédé selon la revendication 14 ou 15, dans lequel l'enzyme est choisie dans le groupe comprenant l'alcool-déshydrogénase, la peroxydase et la phosphatase alcaline. 30
19. Procédé selon la revendication 14, dans lequel le marqueur détectable est choisi dans le groupe comprenant la ferritine, la biotine, l'avidine et la streptavidine, et leurs dérivés. 35
20. Procédé selon la revendication 14 ou 15, dans lequel le colorant est choisi dans le groupe comprenant le bleu Evans et le bleu brillant de Coomassie. 40
21. Procédé selon l'une quelconque des revendications 1 à 20, dans lequel les cellules apoptotiques sont isolées par utilisation d'un cytomètre d'écoulement ou d'une analyse immunologique à détection d'anticorps. 45
22. Procédé selon l'une quelconque des revendications 1 à 20, dans lequel l'apoptose est mesurée sur une plaque de microtitrage pourvue de cellules adhérentes. 50
23. Procédé selon l'une quelconque des revendications 1 à 20, dans lequel les cellules apoptotiques sont séparées des cellules non apoptotiques par utilisation d'une phase solide qui présente une grande affinité pour le réactif qui est fixé à la cellule apoptotique.
24. Procédé selon l'une quelconque des revendications 1 à 20, dans lequel les cellules apoptotiques sont séparées des cellules non apoptotiques par utilisation d'une phase solide qui présente une grande affinité pour l'annexine ou une forme conjuguée de l'annexine. 55
25. Procédé pour déterminer l'effet d'un composé ou d'un traitement spécifique sur le degré d'apoptose, chez un individu et/ou dans un échantillon, qui consiste à mettre en oeuvre le procédé selon l'une quelconque des revendications précédentes, avec un échantillon qui a été soumis à la présence du composé et/ou au traitement spécifique devant faire l'objet de l'essai, et à comparer le résultat au résultat obtenu quand on met en oeuvre le procédé selon les revendications précédentes dans les mêmes conditions avec un échantillon étalon et/ou un échantillon prélevé avant la présence du composé et/ou du traitement spécifique devant faire l'objet de l'essai.
26. Trousse convenant à la mise en oeuvre du procédé selon l'une quelconque des revendications précédentes, comprenant un réactif ayant une constante de dissociation vis-à-vis de la phosphatidylsérine $K_d < 10^{-6} \text{ M}$, et qui est détectable ou peut être rendu détectable, ladite trousse comprenant en outre un marqueur pour distinguer les cellules ayant subi une lyse des cellules intactes.
27. Trousse selon la revendication 26, dans laquelle le marqueur est l'iodure de propidium.
28. Trousse selon la revendication 26 ou 27, qui comprend en outre un cation.
29. Trousse selon l'une quelconque des revendications 26 à 28, qui comprend en outre des milieux appropriés.
30. Trousse selon l'une quelconque des revendications 26 à 29, dans laquelle le marqueur est sélectionnable de façon à permettre la séparation des cellules ayant subi une lyse et des cellules intactes.
31. Utilisation du procédé selon l'une quelconque des revendications 1 à 22, et/ou d'une trousse selon l'une quelconque des revendications 26 à 30, pour séparer des cellules inutilisables les cellules convenant à une transplantation.

32. Utilisation d'un procédé selon la revendication 31, pour séparer les cellules pour transplantation, les cellules étant des cellules médullaires ou des cellules souches autologues ou hétérologues.

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fig-1

WITHOUT CYTOKINES

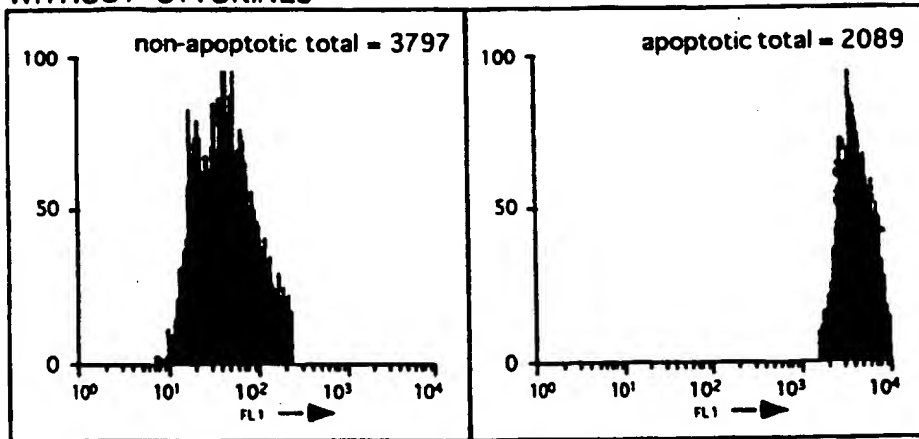


fig-2

WITH CYTOKINES

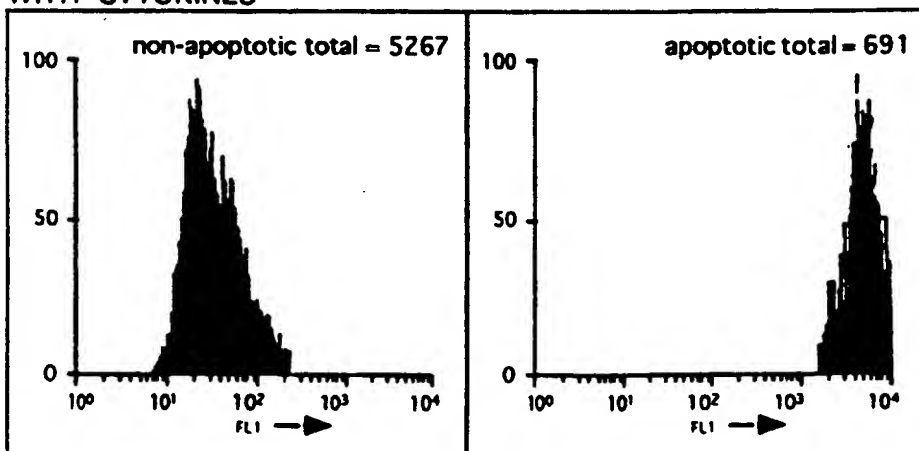


fig-3

